Abnormal Muscle Afferent Function in a Model of Taxol-Chemotherapy-induced Painful Neuropathy

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ABSTRACT

In spite of muscle pain being a well-described symptom in patients with diverse forms of peripheral neuropathy, the role of neuropathic mechanisms in muscle pain have received remarkably little attention. We have recently demonstrated in a well-established model of chemotherapy-induced painful neuropathy (CIPN), that the anti-tumor drug paclitaxel (Taxol®) produces mechanical hyperalgesia in skeletal muscle, of similar time course to and with shared mechanism with cutaneous symptoms. In the present study we evaluated muscle afferent neuron function in this rat model of CIPN. The mechanical threshold of muscle afferents in rats exposed to paclitaxel was not significantly different from the mechanical threshold of muscle afferents in control animals (P=0.07). However, paclitaxel did produce a marked increase in the number of action potentials elicited by prolonged suprathreshold fixed intensity mechanical stimulation, and also a marked increase in the conduction velocity. In addition, the interspike interval (ISI) analysis (to evaluate the temporal characteristics of the response of afferents to sustained mechanical stimulation), showed a significant difference in rats treated with paclitaxel; there was a significantly greater percentage ISI of paclitaxel-treated muscle afferents with 0.01 and 0.02 s interspike interval. In contrast, an analysis of variability of neuronal firing over time (CV2 analysis) showed no effect of paclitaxel administration. These effects...
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of paclitaxel on muscle afferent function contrast with the previously reported
effects of paclitaxel on the function of cutaneous nociceptors.

Keywords: Paclitaxel; Skeletal muscle; Hyperalgesia; Peripheral neuropathy; Sensory afferents; Conduction velocity
INTRODUCTION

While the concept of neuropathic muscle pain has received remarkably little attention, a number of reports describe muscle pain as a prominent symptom in patients with diverse forms of peripheral neuropathy (Bradley et al., 1970, Gardner, 1972, Kunitoh et al., 1998, Marchettini et al., 2006, Peltier & Russell, 2006, van de Glind et al., 2007, Vittadini et al., 2001). And yet, the muscle pain experienced by these patients can be incapacitating, and resistant to treatment (Jacobson et al., 2003). Basic and clinical research on chemotherapy-induced peripheral neuropathy (CIPN), which has focused on nociceptors that innervate the cutaneous domain (Costigan et al., 2009, Baron, 2009), has revealed much about underlying mechanisms. In contrast, very little is known about the muscle pain produced by the same insult to the peripheral nervous system.

Paclitaxel (Taxol®), an antineoplastic agent used to treat various types of cancer (Cavaletti et al., 1995, Chaudhry et al., 1994, Rowinsky et al., 1993, Socinski et al., 2002, Vaishampayan et al., 1999), induces cytotoxicity by promoting stabilization of tubulin polymers, resulting in microtubule dysfunction (Arnal & Wade, 1995, Authier et al., 2000b, Cavaletti et al., 1995, Cavaletti et al., 1997, Schiff & Horwitz, 1980). It also induces painful peripheral neuropathy (Tanner et al., 1998a, Tanner et al., 1998b) as a major dose-limiting
side effect; the incidence of this form of peripheral neuropathy is often greater than 50%, and approaches 90% with some dosage regimens (Rowinsky, 1993, Cavaletti et al., 1995). Paclitaxel containing chemotherapeutic regimens often also produce a pain syndrome characterized by intense myalgias (Jacobson et al., 2003, Kunitoh et al., 1998), which can persist for months (Kunitoh et al., 1998). We recently demonstrated, in an established model of paclitaxel CIPN, persistent muscle pain, manifest as mechanical hyperalgesia, comparable in time course to, and sharing underlying mechanisms with paclitaxel-induced cutaneous mechanical hyperalgesia (Alvarez et al., 2011). The rapid onset and time to peak symptoms in this model of paclitaxel CIPN is consistent with the myalgias reported by patients, which usually begins a few days after its administration, and rapidly reaches an intensity requiring opioid treatment (Jacobson et al., 2003; Loprinzi et al., 2007). To better understand the neuropathic changes in muscle sensory afferents underlying this pain syndrome, in the present study we evaluated muscle sensory afferent function in a rat model of paclitaxel CIPN (Alessandri-Haber et al., 2004, Dina et al., 2001, Dina et al., 2004, Tanner et al., 1998a, Tanner et al., 1998b).
METHODS

Animals

Experiments were performed on 250-400 g adult male Sprague Dawley rats (Charles River, Hollister, CA). Animals were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12 h light/dark cycle and environmentally controlled conditions (lights on 7 am–7 pm; ambient room temperature, 21°–23°C) with food and water available ad libitum. Animal care and use conformed to National Institutes of Health guidelines and measures were taken to minimize pain and discomfort. A total of 40 and 31 fiber were evaluated from 33 control and 19 paclitaxel-treated rats, respectively; n values refer to numbers of individual fibers. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco.

Drugs

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Paclitaxel-induced neuropathy
Paclitaxel was administered as previously described (Dina et al., 2001; Polomano et al., 2001); because of its poor aqueous solubility, it was formulated in a vehicle composed of absolute ethanol and Cremophor EL® (1:1) (BASF, Mt. Olive, NJ), at a concentration of 1 mg/ml. A final concentration of 1 mg/ml was made by adding sterile NaCl (0.9%) just prior to injection. Rats were injected intraperitoneally with paclitaxel (1 mg/kg) on days 0, 2, 4 and 6. Using this protocol, rats demonstrate robust mechanical hyperalgesia in the gastrocnemius muscle, for at least 14 days (Alvarez et al., 2011), the period during which muscle sensory afferent function was evaluated in the present study.

**Single fiber electrophysiology**

The *in vivo* single fiber electrophysiology technique employed was similar to that used previously in recordings from cutaneous afferents (Chen et al., 1999). Rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, i.p., with additional doses given throughout the experiment to maintain areflexia), their trachea cannulated, and heart rate monitored. Body temperature was maintained at 37 ± 0.5 °C, using a heating blanket regulated by a rectal temperature sensor. Anesthetized animals were positioned on their right side and an incision made on the dorsal skin of the left leg, between the mid-thigh and calf, and the biceps femoris muscle partially removed to expose the sciatic nerve and gastrocnemius muscle. The edges of the incised skin were fixed to a metal loop to provide a pool...
that was filled with warm mineral oil to bathe the sciatic nerve and gastrocnemius muscle.

The sciatic nerve was cut proximally to prevent flexor reflexes during electrical stimulation of sensory neurons. Fine fascicles of axons were then dissected from the distal stump, and placed on a recording electrode. Single units were first detected by mechanical stimulation of the gastrocnemius muscle with a small blunt-tipped glass bar. Bipolar stimulating electrodes were then placed and held on the center of the receptive field of the muscle afferent, by a micromanipulator (MM-3, Narishige, Japan). Conduction velocity of each fiber was calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked action potential. All recorded muscle afferents had conduction velocities in the range of type III (12%) or type IV (88%) fibers (Diehl et al., 1993); muscle afferents are grouped by conduction velocity: ~2.5 – 30 m/s for group III and <2.5 m/s for group IV (Berberich et al., 1988). To exclude the possibility of mechanical activation of afferent fibers when applying electrical stimulation for determination of conduction velocity, repeated electrical stimuli were delivered to confirm the electrically evoked action potential, which also helps exclude mechanical activation of afferent fibers. Mechanical threshold was determined with calibrated von Frey hairs (VFH Ainsworth, London, UK) and defined as the lowest force that elicited at least 2 spikes within 1 s, in at least 50% of trials. Sustained (60 s) suprathreshold
(10 g) mechanical stimulation was accomplished by use of a mechanical
stimulator that consisted of a force-measuring transducer (Entran, Fairfield, NJ,
USA) with a blunt plastic tip that was applied by a micromanipulator (BC-3 and
BE-8, Narishige) on the center of the receptive field, for 60 s. We did not
systematically analyze the spontaneous discharge. Neural activity and timing of
stimulus onset and termination were monitored and stored on a Windows OS
computer with Micro 1401 interface (CED, Cambridge, UK) and analyzed off-line
with Spike2 software (CED).

**Interspike interval (ISI) analysis**

ISI analysis, used to evaluate the temporal characteristics of the response
of nerve fibers to sustained mechanical stimulation, was adopted from our study
of afferent activity in the rat model of vincristine-induced painful neuropathy
(Tanner et al., 2003). The ISIs for the responses of each afferent were grouped
into 100 ms bins between 0 and 499 ms; the few ISIs greater than or equal to 500
ms were not further analyzed (Tanner et al., 2003). This bin width also allows
comparison of data with that from previous studies (Franck et al., 1993, Miller &
Woolf, 1996, Arendt-Nielsen et al., 2000). The number of intervals occurring in
each bin was expressed as the percentage of the total number of ISIs in the trial.
This normalization procedure allowed the distribution of ISIs from several fibers
to be averaged together.
Coefficient of variation analysis

ISIs do not give an accurate estimate of the variability of neuronal firing if the mean firing rate changes over time, a common occurrence. Therefore, we also calculated the coefficient of variability (CV2) which compares the relative difference between adjacent ISIs (Holt et al., 1996). CV2 is defined as the square root of 2 multiplied by the S.D. of two ISIs divided by their mean (Holt et al., 1996):

\[ CV_2 = \sqrt{2} \frac{|\Delta t_{i+1} - \Delta t_i|}{\Delta t_{i+1} + \Delta t_i}, \] where \( t_i \) is the latency for the \( i \)th action potential.

Thus, CV2 is a dimensionless number that is independent of absolute firing rate.

That differences in CV2 reflect physiologically meaningful differences between functionally important classes of neurons were recently demonstrated in a study separating slowly adapting type I from type II afferents fibers (Wellnitz et al., 2010).

Statistical analyses

Group data are expressed as mean ± SEM of \( n \) distinct observations. Statistical comparisons were made by Student’s t-test (for one or two
independent populations) or by one-way ANOVA for comparing multiple treatments (GraphPad Prism statistical software). Data was tested for normality using the D’Agostino and Pearson omnibus normality test; if data did not pass the normality test for Gaussian distribution, Welch’s correction for the Student’s t-test was used (GraphPad Prism software). To compare change from baseline, one-way repeated-measures ANOVAs with a Greenhouse-Geisser adjusted $P$-value was used (SPSS statistical software). To compare CV2 analyses, a one-way repeated-measures ANOVA was used. $P < 0.05$ was considered statistically significant.
**RESULTS:**

*Mechanical threshold*

We first evaluated the effect of paclitaxel treatment on the mechanical threshold of afferents in the gastrocnemius muscle of the rat. While the mechanical threshold of muscle afferents exposed to paclitaxel (0.89± 0.09 mN, n= 31) was lower than the mechanical threshold of muscle afferents in naïve control animals (1.11±0.11 mN, n = 40; Fig. 1), this difference did not reach statistical significance (P=0.07).

*Response to sustained stimulation*

We next evaluated the effect of paclitaxel treatment on the response of afferents to a uniform intensity, sustained suprathreshold mechanical stimulus. The response of muscle afferents to a 60 sec suprathreshold (10 g) mechanical stimulus in paclitaxel treated rats (431.80 ± 93.46 action potentials/60 sec stimulus, n= 31) was significantly greater than in muscle afferents from naïve control animals (215.80 ± 49.91 action potentials/60 sec stimulus, n= 40, P = 0.047, Students t-test, Welch’s correction, Fig. 2).

*Afferent firing pattern/variability*
Since we have previously observed changes in firing pattern in afferents in muscle (Chen et al., 2010) and skin (Chen & Levine, 2003, Tanner et al., 2003), in models of painful peripheral neuropathy, we evaluated firing pattern in muscle afferents in rats treated with paclitaxel. To examine the pattern of neural activity, we first generated inter-stimulus interval (ISI) histograms and performed coefficient of variation (CV2) analyses for muscle afferents recorded in paclitaxel treated and control rats.

The peak in the ISI distribution of muscle afferents from paclitaxel-treated rats was shifted to the left, compared to afferents from control animals, reflecting the greater number of action potentials elicited by the 10 g von Frey stimulus in afferents from paclitaxel-treated rats. In rats treated with paclitaxel, there was a significantly greater percentage ISI of paclitaxel-treated muscle afferents with 0.01 and 0.02 s interspike interval and significantly smaller percentage with those with >2.9 s interspike interval (two-way repeated measures ANOVA, with Bonferroni post hoc test, P<0.05, control vs. paclitaxel exposed, Fig. 3). Although we found no difference in CV2 values for muscle afferents in paclitaxel-treated compared to control rats (data not shown), in addition to substantial increase response to sustained suprathreshold stimulation in muscle afferents from paclitaxel treated rats, there was also a change in firing patterns in these afferents.

Conduction velocity
In paclitaxel-treated rats, the conduction velocity of muscle afferents (2.09 ± 0.17 m/sec, n = 31) was significantly faster than that of afferents in control animals (1.25 ± 0.11 m/sec, n = 40, Student’s t-test, with Welch’s correction, P<0.0001; D’Agostino and Pearson omnibus normality test for control fibers, P=0.006 does not pass normality test; paclitaxel-treated fibers, passes normality test) (Fig. 4). The frequency distribution of conduction velocities in fibers of paclitaxel-treated and control fibers indicates a shift in the distribution of conduction velocity, with a higher percentage of faster-conducting fibers in NLB rats (Fig. 5). Thus, while not excluding a contribution of a difference in percentage of type III and type IV fibers that were sampled in the paclitaxel treated versus control groups of rats, the shift in distribution is most compatible with the suggestion that our findings are explained by an increase in conduction velocity induced by paclitaxel in muscle afferents. While it is possible that paclitaxel treatment has a preferential effect on a subset of neurons, e.g. more superficial neurons, leaving a greater proportion of deeper terminals that may require more force to be activated, our methodology does not distinguish the superficiality of a particular fiber’s receptive field. However, given the relatively large increase in conduction velocity (~67%), it is likely that at least some of the observed difference is due to changes in conduction velocity in the axon. Furthermore, the average mechanical threshold is not significantly different between the paclitaxel-treated and control groups, supporting the idea that we are not sampling a different population of fibers in the experimental and control
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animals. Finally, we have recently shown a similar change in another model of

muscle pain (Chen et al., 2011).
DISCUSSION

While as a clinical entity, neuropathic muscle pain has been largely neglected, many forms of peripheral neuropathy manifest persistent, sometimes debilitating, and often difficult to treat pain in skeletal muscle (Bradley et al., 1970, Gardner, 1972, Kunitoh et al., 1998, Marchettini et al., 2006, Peltier & Russell, 2006, van de Glind et al., 2007, Vittadini et al., 2001). We have recently observed mechanical hyperalgesia in skeletal muscle, in three models of painful peripheral neuropathy, namely CIPN induced by two chemotherapeutic drugs that have CIPN as dose-limiting side effects, paclitaxel- (Alessandri-Haber et al., 2004, Dina et al., 2001, Dina et al., 2004) and oxaliplatin- (Ferrari et al., 2010, Joseph et al., 2008, Joseph & Levine, 2009) induced CIPN, and the painful peripheral neuropathy associated with chronic alcohol consumption (Dina et al., 2000, Dina et al., 2007, Dina et al., 2008), where the mechanical hyperalgesia in these forms of painful peripheral neuropathy have a time course that parallels cutaneous hyperalgesia in the same animal, and share mechanisms in common (Alvarez et al., 2011).

In the present study we evaluated the function of muscle afferents in rats with one of these forms of neuropathy, paclitaxel-induced painful peripheral
Taxol sensitizes muscle afferents neuropathy. Although paclitaxel treatment did not produce a significant change in mechanical threshold, a marked increase in number of action potentials induced by a sustained (60 sec) suprathreshold (10 g) mechanical stimulus, and a marked increase in conduction velocity, both of which were statistically significant. Using ISI and CV2 analyses, we demonstrated the variability in evoked activity in muscle afferents.

These present findings of the effects of paclitaxel on muscle afferents contrast markedly with those in our previous study of cutaneous C-fiber function in rats with paclitaxel-induced CIPN (Dina et al., 2001). In that study, we found no significant difference in mechanical threshold, response to sustained suprathreshold mechanical stimulation or conduction velocity in C-fibers innervating the skin on the dorsum of the rat’s hind paw. Although the mean number of action potentials evoked by a sustained (60-s) threshold and suprathreshold (10-g) stimulus was higher for afferents from paclitaxel-treated animals than in those from control rats, the difference did not reach statistical significance, similar to what we have previously observed in models of diabetic- (Ahlgren & Levine, 1994, Chen & Levine, 2001), vincristine- (Tanner et al., 1998b) and alcohol- (Dina et al., 2000) induced painful peripheral neuropathy. However, as in these other models, paclitaxel treatment produced a subpopulation of cutaneous C-fibers with increased number of action potentials evoked by
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sustained (60 s) threshold and suprathreshold (10 g) stimulation of cutaneous C-fibers. The high-firing-frequency C-fibers had approximately a three-fold higher firing rate compared to controls during a 60-s stimulus while the low-firing-frequency fibers had responses similar to those of C-fibers in control rats. Of note, this isolated population of afferents, with very high response rates, was not observed in the present study of muscle afferent function in paclitaxel-induced CIPN in the rat. The reasons for these marked differences in the effect of paclitaxel on afferent function in muscle compared to skin, is currently unknown. Unfortunately, compared to the many detailed analyses of the cell biology of the cutaneous afferent, our knowledge of the cell biology of muscle afferent is still very rudimentary.

The finding that the conduction velocity in muscle afferents was increased by paclitaxel was unexpected, not only because of our prior finding that paclitaxel did not produce a change in conduction velocity in cutaneous afferent, but also because in the setting of peripheral neuropathy, if changes in conduction velocity are observed, they have usually been reported to be a decrease in conduction velocity. While in the setting of hypersensitivity states, one might expect to observe an increase in conduction velocity, as a reflection of a change in ionic conductance in the axon (Hodgkin, 1975, Waxman et al., 1999), in the setting of inflammatory pain, conduction velocity has generally been unchanged.
Taxol sensitizes muscle afferents (Nakatsuka et al., 1999, Baba et al., 1999), while in peripheral neuropathies, even those associated with pain, changes when observed have in general been in the form of a slowing in conduction velocity (Elliott et al., 2009, Meyer et al., 2010, Nakatsuka et al., 1999). These paradoxical findings may be due, in part, to the fact that clinically, in patients with peripheral neuropathies (Truini et al., 2009, Nardone & Schieppati, 2004, Shefner et al., 1991), and in animal models of neuropathic pain (Cermenati et al., 2010, Jolivalt et al., 2009, Brussee et al., 2008, Meyer et al., 2010, Authier et al., 2000a), conduction velocity is generally measured in myelinated, non-nociceptive, afferents. However, there are also studies in which slowing of C-fiber conduction velocity in neuropathic pain models (e.g., ddC (Chen & Levine, 2007)), and in clinical studies on cutaneous unmyelinated fibers patients with neuropathic pain (e.g., erythromelalgia (Orstavik & Jorum, 2010)) have been reported. Methodological differences may also play a role, since while Baba and colleagues observed no change in C-fiber conduction velocity when complete Freund’s adjuvant was injected in the rat’s hind paw (Baba et al., 1999), Dhjouri and Lawson (Djouhri & Lawson, 2001), observed increased conduction velocity after complete Freund’s adjuvant was injected into the limb as well as the paw. Thus, direct exposure of the peripheral nerve to the neuropathic effect of cytokines may produce enhancement of conduction velocity. Possibly related to this, Gold and colleagues have recently shown that persistent inflammation alters the density and distribution of voltage-activated ion channels in DRG neurons (Lu et al., 2010). Alternatively,
complete Freund’s adjuvant may have different effects on cutaneous afferents and those innervating deep tissues (e.g. muscle, tendon or bone). In support of this, we have recently demonstrated that water avoidance stress, which produces mechanical hyperalgesia in muscle, produced changes in muscle afferent function very similar to those induced by paclitaxel, including significant increase in conduction velocity (Lu et al., 2010). While the underlying mechanism(s) responsible for the increased conduction velocity in muscle afferents is unknown, they are likely due to changes in ionic conductances in the afferent axon (Quasthoff, 1998). Paclitaxel-induced changes in ion channel function will be evaluated in future studies.

In conclusion, we have shown that persistent muscle hyperalgesia in a rat model of paclitaxel chemotherapy-induced painful neuropathy (Alessandri-Haber et al., 2004, Dina et al., 2001, Dina et al., 2004, Tanner et al., 1998a, Tanner et al., 1998b) is associated with enhanced activity in muscle afferents. These changes are markedly different from those previously reported for the effect of paclitaxel on cutaneous afferent function (Alessandri-Haber et al., 2004, Dina et al., 2001, Dina et al., 2004). The cell biological basis of the differences between muscle and cutaneous afferents responsible for the different effects of paclitaxel on the function of these two types of afferents remain to be elucidated.
Disclosure

The authors of this manuscript have no conflicts of interest with regard to publishing these data.
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Figure legends

Figure 1. Mechanical threshold of muscle afferents

Mechanical threshold in afferents innervating the gastrocnemius muscle of paclitaxel-treated rats were not significantly different from the threshold in afferents from naïve control rats. Scattergram of mechanical thresholds of individual muscle afferents from naïve control and paclitaxel-treated rats is also shown.

Figure 2. Response of muscle afferents to a sustained mechanical stimulus

The responses of afferents innervating the gastrocnemius muscle of paclitaxel-treated rats to mechanical stimuli (60 s suprathreshold 10 g von Frey hair, n = 31) were significantly higher than those of control rats (n = 40, *P<0.05). Scattergram of individual responses of muscle afferents from naïve control and paclitaxel-treated rats is also shown.

Figure 3. Inter-stimulus interval (ISI) distribution of muscle afferents in response to sustained mechanical stimuli
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No significant differences were observed in the ISI distribution of muscle afferents (from 0 – 0.28 s, in 0.01 s bin width, and >0.29 s), from control and paclitaxel-treated rats, in response to mechanical stimulation (60 s suprathreshold 10 g von Frey hair).

Figure 4. Conduction velocity in muscle afferents

Mean conduction velocities from paclitaxel-treated rats (2.09 ± 0.17 m/s, n=31) were significantly greater than from naïve rats (1.25 ± 0.11 m/s, n=40, Student’s t-test, p < 0.05). Scattergram of conduction velocities of muscle afferents in naïve control and paclitaxel-treated rats is also shown.

Figure 5. Paclitaxel treatment stress shifts muscle afferent conduction velocity frequency distribution

A plot of frequency distribution of conduction velocity indicates that there is a shift to faster conducting fibers in paclitaxel-treated rats. Graph overlay shows smoothing curve of data (6th order polynomial, 4 neighbor averaging) to illustrate shift in conduction velocity distribution.
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Mechanical threshold

Figure 1
Figure 2

Spike count

Spikes/60 seconds

Control       Paclitaxel

0               200
200             400
400             600
600             800
800             1000
1000            1500
1500            2000
2000            2500

*
Figure 3

![Graph showing interspike interval (s) and ISI (%) for Control and Paclitaxel conditions. The graph includes error bars and asterisks indicating statistical significance.](image-url)
Conduction velocity

Figure 4
Conduction velocity frequency distribution

Figure 5